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14. ABSTRACT

Bone metastasis is one of the major causes of morbidity and mortality in breast cancer (BC) patients. However, only a few human BC cell lines can efficiently metastasize to bone whereas most BC cell lines cannot. Recently, it was shown that systemic administration of the conditioned medium by a melanoma cell line redirected the metastatic dissemination of a weakly metastatic lung carcinoma cell line to the organ sites that were metastasized by the melanoma cell due to the formation of metastasis-permissive niches by bone marrow-derived cells (BMDCs) in these organ sites. The current project was proposed to test the hypothesis that factors secreted by metastasis-competent BC cells may condition bone marrow for the successful homing and skeletal remodeling of circulating BC cells. Athymic nude mice were treated with media conditioned by metastasis-competent BC cells or inoculated orthotopically with metastasis-competent BC cells and intracardiacally with non-metastatic BC cells. Non-metastatic BC cells were found in bone of mice bearing orthotopic BC tumors.

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Introduction

Bone metastasis is one of the major causes of morbidity and mortality in breast cancer (BC) patients. While several molecular pathways have been shown or implicated to promote bone metastasis of human BC cells, how metastatic BC cells specifically take residence in skeletal tissue remains unknown.

It is widely believed that bone provides an optimal environment for BC cells to adhere and proliferate, which is the “seed-and-soil” hypothesis. However, only a few human BC cell lines can readily metastasize to bone when inoculated in the arterial circulation in mice. For example, human BC MDA-MB-231 cells cause noticeable osteolytic bone metastasis in 4 weeks whereas it takes about 6 months for human BC ZR-75-1 cells to cause significant osteoblastic bone metastasis when the cells were injected in the left ventricle of female nude mice (1). Human BC MDA-MB-435 cell and its variant metastasize to bone in 4-5 weeks in the same animal model system, but caused very limited osteolysis (2). Thus, the modulation of the “soil” by different “seeds” may determine the efficacy of the “seeding” as well as the subsequent remodeling of the “soil”.

Recently, Kaplan et al. (3) showed that systemic administration of the conditioned medium by a melanoma cell line redirected the metastatic dissemination of a weakly metastatic lung carcinoma cell line to the organ sites that were metastasized by the melanoma cell, not by the lung carcinoma cell. This was due to the formation of metastasis-permissive niches by bone marrow-derived cells (BMDCs) in these organ sites after the treatment with the melanoma cell-conditioned medium. The BMDCs then attracted the lung carcinoma cells to these organ sites. Thus, this new concept suggests that tumor cell-secreted factors can initiate site-specific construction of metastasis-permissive niches before the arrival of metastatic cells. On the basis of these observations, we hypothesized that factor(s) secreted by metastasis-competent BC cells may condition bone marrow for the successful homing and skeletal remodeling of circulating BC cells. Our initial study indicated that the media conditioned by metastasis-competent BC cells had limited effect on bone metastasis of metastasis-incompetent cells. Therefore, we investigated whether the presence of orthotopic mammary tumors formed by metastasis-competent BC MDA-MB-231 cells will enhance bone metastasis of circulating BC ZR-75-1 cells that do not metastasize to bone by themselves in the absence of estrogen supplement. Our results indicate that the presence of malignant mammary tumors can induce metastasis-incompetent ZR-75-1 breast tumor cells to home to bone. We also report a hybrid human breast cancer cell line through the fusion of human breast cancer MDA-MB-231 and ZR-75-1 cells in bone marrow.

Body

1. The presence of malignant orthotopic breast tumors promoted homing of non-metastatic breast cancer cells to bone.

Five-week-old athymic nude female nude mice were inoculated orthotopically in the mammary fat pad area with estrogen receptor alpha (ERα) negative MDA-MB-231/GFP/Neo or MDA-MB-435/GFP cells. ERα positive human breast cancer ZR-75-1/GFP/puro cells were then injected intracardiacally into mice bearing growing tumors formed by MDA-MB-231 or MDA-MB-435-F-L cells. We found ZR-75-1/GFP/puro cells in bone marrow of mice with growing tumors formed by MDA-MB-231/GFP/Neo and MDA-MB-435/GFP cells in the absence of estrogen supplementation, but not in control mice without growing orthotopic tumors. The metastatic ZR-75-1/GFP/puro cells were flushed out of the bone marrows and established as variant cell lines. Two variant lines, called B4 and B6, from two mice bearing MDA-MB-435/GFP or MDA-MB-231/GFP/Neo tumors respectively were further characterized for their tumorigenicity in the mouse mammary fat pad and their metastatic potential to lung and bone through intracardiac injection in the presence or absence of estrogen supplementation. Surprisingly, although both variants were isolated from bone marrow and they showed the same anchorage-dependent growth property in vitro (Fig. 1), B6 was significantly more tumorigenic (Fig. 2) and metastatic than B4 in both with or without estrogen supplementation in vivo (Table 1). In fact, B6 was equally or more tumorigenic and metastatic to bone, lung and brain in the absence of estrogen supplementation than B4 in the presence of estrogen supplementation. Like parental ZR-75-1 cell, B4 is not tumorigenic and metastatic without estrogen supplementation.

2. Isolation and characterization of a highly metastatic hybrid cell line generated between ER negative and ER positive breast cancer cells in mouse bone marrow

Because B6 cells were tumorigenic in an estrogen-dependent and –independent manner (Fig. 2), we established two cell lines from the tumors formed by B6 cells. The cell line established from an estrogen-dependent tumor was called B6TE and the cell line established from an estrogen-independent tumor was called B6TC. Further characterization revealed that B6TC cell was more tumorigenic without estrogen supplementation than with estrogen supplementation, which is opposite to the effect of estrogen on the growth of tumors formed by B6TE (Fig. 3). More interestingly, B6TC cell was found to be resistant to both puromycin and G418 suggesting that it was derived from the fusion of MDA-MB-231/GFP/Neo and ZR-75-1/GFP/puro in the mouse bone marrow. When compared with its parent MDA-MB-231/GFP/Neo, B6TC cells were found to be more metastatic after intracardiac inoculation (Fig. 4). Bone and lung metastasis detected by whole mouse GFP imaging revealed that in B6TC group, eight out of ten mice developed metastases to lung and bone and seven mice became paralyzed in the hind limbs while in the MDA-MB-231/GFP/Neo group, only six out of ten mice developed lung and bone metastases and three of them became paralyzed at the end of fourth week. More significantly, three of B6TC mice also developed metastases to brain (Fig. 5), which was not observed in the MDA-MB-231/GFP/Neo group. B6TC cell line has a low expression of ER alpha and CD24, and high expression of CD44 and ALDH (Fig. 6) suggesting that the cell line contains a high percentage of stem-like cancer cells. It also expresses vimentin, CXCR4 and Integrin-β1 (Fig. 6). Its growth is refractory to the anti-estrogens, Raloxifene (Fig. 7).

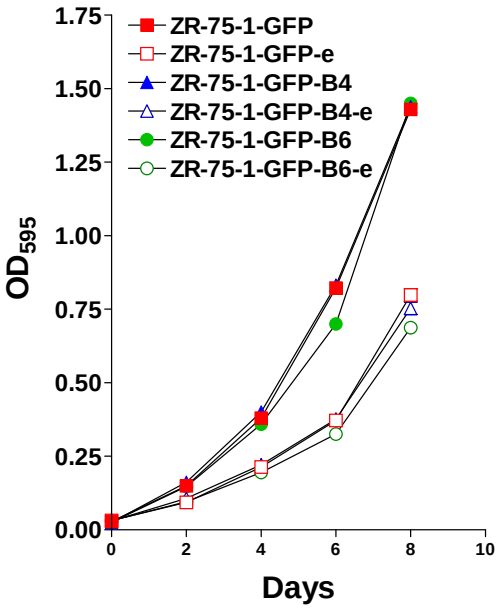

Fig. 1.Growth curve of GFP-expressing parental ZR-75-1 cells, B4 and B6 cells. The cells were plated at 1,500 cells/well in a 96-well plate in complete or estrogen deficient (-e) medium. MTT assay was used to obtain relative cell number as optical density (OD) at the depicted time points. Each value is the mean+SEM from 4 wells.

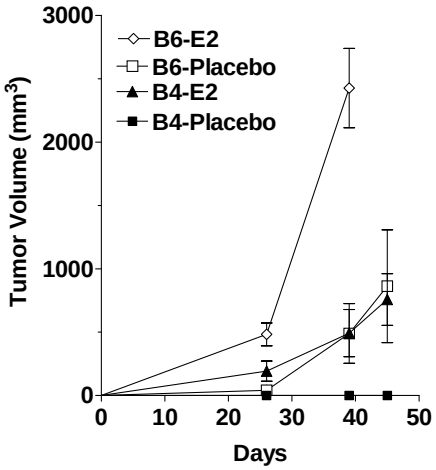

Fig. 2. Xenograft growth curve. Exponentially growing GFP-expressing B4 and B6 cells ($2X10^6$) were inoculated into both inguinal mammary fat pads of female nude mice. Two groups of animals (n=5) were implanted with either 17β estradiol (E2) pellets (0.72 mg/90 days) or placebo pellets (Innovative Research of America, FL). Xenografts were measured externally in two dimensions using a caliper. Tumor volumes were calculated with the equation $V = (L \times W^2) \times 0.5$, where L is length and W is width of a tumor. Each value is the mean±SEM of summed tumor burden in 5 mice.

Table 1. Metastatic incidence in bone, lung and brain*

Group	Bone metastasis		Lung metastasis detected by GFP imaging	Brain metastasis detected by GFP imaging
	Whole mouse GFP imaging	Histological staining		
	Incidence	Tumor incidence in femora-tibiae	Incidence	Incidence
B4-Placebo	0/5$	0/10$$	0/5	0/5
B4-E2	1/4	1/8	3/4	1/4
B6-Placebo	3/5	4/10	4/5	1/5
B6-E2	5/5	6/10	5/5	2/5

*In an intra-cardiac injection model, GFP-expressing B4 and B6 cells were injected into the left cardiac ventricle of anesthetized 4-week-old female nude mice implanted with either 17β estradiol (E2) pellets (0.72 mg/90 days) or placebo pellets with a 27-gauge needle attached to a 1-ml syringe using a micromanipulator. Successful injections were indicated by the pumping of red blood into the syringe. After 8 weeks, metastases in the femur/tibia were detected by whole mouse GFP imaging and by histological staining. Metastases in the lung and brain were detected in the excised organs by GFP imaging.

$ indicates 0 out of 5 mice.

$$ indicates 0 out of 10 femora-tibiae.

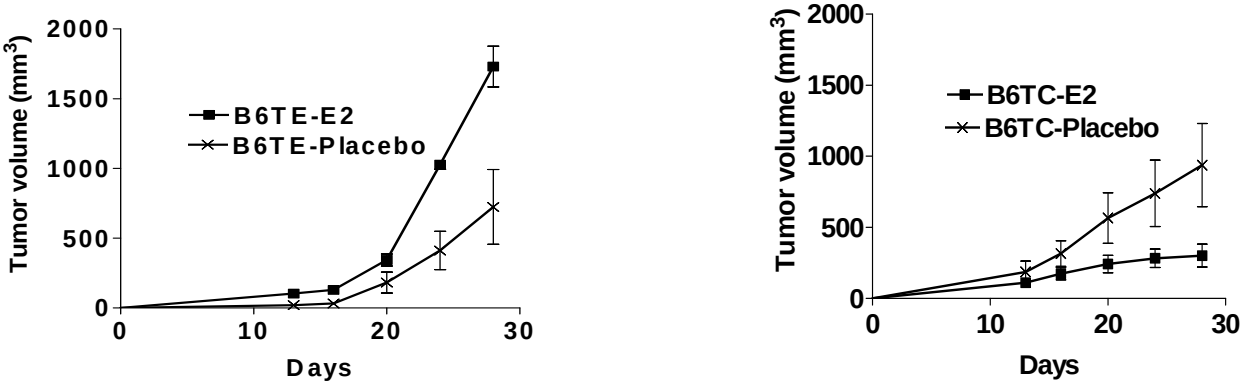

Fig. 3. Effect of estrogen supplementation on the growth of tumors formed by B6TE or B6TC. Exponentially growing GFP-expressing B6TE and B6TC cells ($2X10^6$) were inoculated into both inguinal mammary fat pads of female nude mice. Two groups of animals were implanted with either 17β estradiol (E2) pellets (0.72 mg/90 days) or placebo pellets. Xenografts were measured externally in two dimensions using a caliper. Tumor volume was calculated as described in the legend of Fig. 2. Each value is the mean±SEM of summed tumor burden in 4 mice for B6TE-E2 group and 5 mice for the other groups.

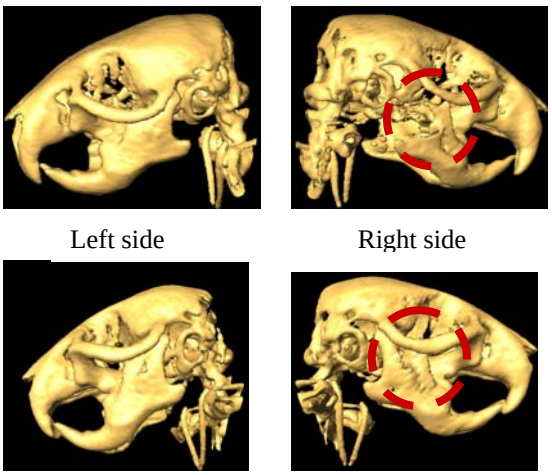

Fig. 4. Osteolytic bone metastasis induced by B6TC or MDA-MB-231 cells. GFP-expressing B6TC and MDA-MB-231 cells were injected into left ventricle of female nude mice. When GFP-tumors were detectable in the jar area of mice after four weeks, the mice were imaged with microCT. Representative images of left and right head bones of one mouse from each group were shown. More extensive bone loss can be seen in the right jar area (red circle) of the B6TC mouse than in the MDA-MB-231 mouse.

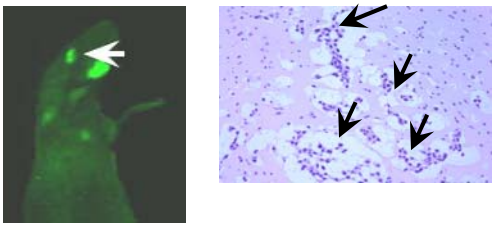

Fig. 5. Brain metastasis induced by B6TC cells. Brain metastatic tumors pointed with arrows were detected with GFP imaging in the left panel and histology in the right panel four weeks after intracardiac injection of B6TC cells.

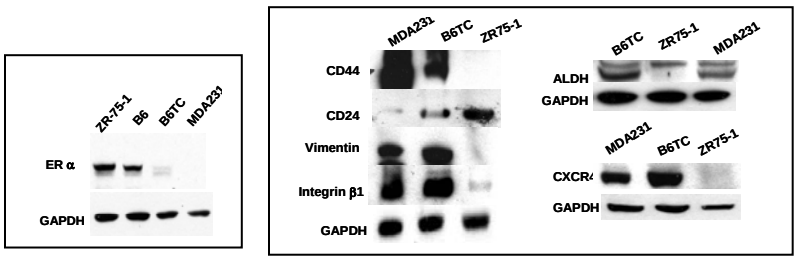

Fig. 6. Comparison of protein expression levels of various genes among cell lines. Western blotting analysis was used to compare the expression of various genes among the cell lines depicted. GAPDH levels were used to indicate equal sample loading.

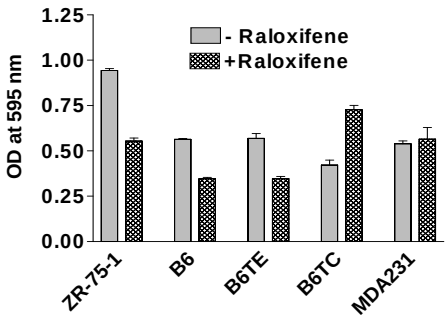

Fig. 7. Effect of Raloxifene on cell growth. Cells were plated at 1,500 cells/well in a 96-well plate and treated with 10^{-7} M Raloxifene for 6 days. Relative cell number was obtained with MTT assay. Values are mean±SEM from 4 wells.

KEY RESEARCH ACCOMPLISHMENTS

1. Obtained a hybrid human breast cancer cell line called B6TC through fusion of MDA-MB-231 and ZR-75-1 cells in mouse bone marrow.
2. Completed study to compare the tumorigenic and metastatic properties among ZR-75-1/GFP/puro, MDA-B-231/GFP/Neo, and B6TC cell lines in athymic female nude mice.
3. A manuscript reporting our findings is under preparation for publication.

REPORTABLE OUTCOMES

Cell line: hybrid human breast cancer cell line called B6TC

Abstract:
Bandyopadhyay, A., Long Wang, Junhua Yang, Keya De, Yuping Tang and LuZhe Sun. Isolation and characterization of bone marrow derived metastatic ZR-75-1 cells in animal models of human breast cancer. DOD Breast Cancer Research Program, Era of Hope Meeting, June 25-28, 2008, Baltimore, MD.

Keya De, A. Bandyopadhyay, J. Yang, L. Wang and L. Z. Sun. Isolation and characterization of a highly metastatic hybrid cell line generated between ER negative and ER positive breast cancer cells in mouse bone marrow. Poster presentation at the 31th Annual San Antonio Breast Cancer Symposium, Dec. 10-14, 2008, San Antonio, TX.

CONCLUSIONS

Our study indicates that the presence of malignant mammary tumors can induce metastasis-incompetent ZR-75-1 breast tumor cells to home to bone. ZR-75-1/GFP/puro/B6 cells generated in the bone marrow were a heterogeneous population of ERα-positive and ERα-negative cells with marked differences in their proliferative potential and malignancy. The low ERα-expressing B6TC is a novel hybrid cell line generated spontaneously in a metastatic site, which has propensity to metastasize to brain in addition to lung and bone. The expression of CD44 and ALDH indicates that it has stem cell-like features. This cell line should be a useful model for the investigation of the molecular mechanism of brain metastasis and the therapeutic strategies for the treatment of metastatic breast cancer resistant to anti-estrogens.

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